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(54) Title: NEW XYLANASES HAVING HIGH ACTIVITY AND STABILITY AT ALKALINE CONDITIONS AND HIGH TEMPERATURES

(57) Abstract

The present invention relates to novel xylanolytic enzymes obtainable from strains of alkalophilic *Bacillus sp.* Moreover, the invention relates to a method for producing the enzymes and the use of the enzymes in the pulp and paper industry.

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NEW XYLANASES HAVING HIGH ACTIVITY AND STABILITY AT ALKALINE CONDITIONS AND HIGH TEMPERATURES

TECHNICAL FIELD

The present invention relates to novel xylanolytic enzymes obtainable from strains of alkalophilic Bacillus sp. Moreover, the invention relates to a method for producing the enzymes and the use of the enzymes in the pulp and paper industry.

BACKGROUND ART

Xylanases with high activity and stability at alkaline conditions are of great commercial interest, e.g. for applications in the pulp and paper industries, for modification of lignocellulose. However, very few xylanases are reported which are able to function at pH values 9-12, and the available literature indicates that these enzymes are rapidly inactivated at a pH of more than 10, especially at temperatures exceeding 50°C.

SUMMARY OF THE INVENTION

The present invention describes new xylanase enzymes obtained from alkaline Bacillus sp., which are superior to previously described bacterial xylanases with respect to activity and stability in the alkaline region. Furthermore, the xylanases of the present invention are also able to function at high temperature, e.g. 70°C at pH 7-8.

Accordingly, the invention provides enzyme preparations having xylanolytic activity, and having more than 50% relative activity in the range pH 6-9 at 50°C and temperature optimum in the range of from 55 to 75°C (at pH 6-10).

In another aspect, the invention provides a process for the preparation of the enzyme preparations comprising cultivation of a strain of Bacillus sp., preferably the strain Bacillus sp., DSM 7197, or a mutant or a variant thereof, in a suitable nutrient medium, containing carbon and nitrogen

sources and inorganic salts, followed by recovery of the desired enzyme.

In a third aspect, the invention relates to the use of the enzyme preparation in a process for treatment of lignocellulosic pulp.

In a further aspect, the invention provides an agent containing an enzyme preparation, provided in the form of a granulate, preferably a non-dusting granulate, a liquid, in particular a stabilized liquid, a slurry, or a protected enzyme.

BRIEF DESCRIPTION OF DRAWINGS

The present invention is further illustrated by reference to the accompanying drawings, in which:

Figs. 1-3 show the temperature profiles of the fraction purified according to Ex. 2, in standard Britton & Robinson buffers at pH 7, pH 9, and pH 10, respectively. All reaction mixtures contained 0.013 EXU/ml and were incubated for 30 minutes (⋈ sample; ▲ substrate blank);

Fig. 4 shows the effect of pH on the activity of the fraction purified according to Ex. 2, in 50 mM Britton & Robinson buffers (0.013 EXU/ml; 30 minutes of incubation; 50°C; ★ sample; ▲ buffer; ⋈ enzyme blank); and

Figs. 5, 6, and 7 show the effect of temperature and pH on the stability of the fraction purified according to Ex. 2, in the absence of substrate. The fraction was diluted to a concentration of 0.05 EXU/ml in 50 mM Britton & Robinson buffers of pH 7, pH 9, and pH 10, respectively, and incubated at 40°C. At appropriate intervals, 50 µl samples were removed from each incubation mixtures and transferred to 950 µl 50 mM Britton & Robinson buffer pH 10. The residual xylanolytic activity was determined at 50°C using Xylazyme Tablets™ (Megazyme, Australia). The incubation time was 30 minutes in all cases (⋈ sample; ▲ blind).

DETAILED DISCLOSURE OF THE INVENTION

The invention provides xylanase preparations having high stability and excellent activity at alkaline conditions.

The enzyme preparation of the invention can be further described by the following characteristics.

Physical-Chemical Properties

The enzyme preparation of the invention comprises at least 5 xylanolytic enzymes, having pI in the range of from appr. 3 to appr. 9.5.

At 50°C the fraction of the enzyme preparation, purified according to Ex. 2, has more than 50% relative activity in the range pH 6-10, determined after 30 minutes of incubation. No pronounced pH optimum is detectable, but appears to be in the range pH 5.5 to 9.0 (cf. Fig. 4).

At pH 7.0 the fraction of the enzyme preparation, purified according to Ex. 2, has a temperature optimum in the range of 60 to 75°C, more specifically around 70°C, determined after 30 minutes of incubation (cf. Fig. 1).

At pH 9.0 the fraction of the enzyme preparation, purified according to Ex. 2, has a temperature optimum in the range of 55 to 75°C, more specifically in the range of 60 to 75°C, determined after 30 minutes of incubation (cf. Fig. 2).

At pH 10.0 the fraction of the enzyme preparation, purified according to Ex. 2, has a temperature optimum in the range of 50 to 70°C, more specifically around 60°C, determined after 30 minutes of incubation (cf. Fig. 3).

The fraction of the enzyme preparation, purified according to Ex. 2, has a relative residual activity after incubation for 6 hours at pH 10 and 40°C of at least 90%, more preferred at least 95%, most preferred at least 99%. A similar relative residual activity was observed after incubation for 6 hours at pH 10 and 55°C, at pH 10 and 50°C; at pH 10 and 40°C; at pH 9 and 40°C; at pH 9 and 50°C; and at pH 7 and 50°C, cf. Ex. 3.

Immunochemical Properties

The enzyme preparation of the invention has immunochemical properties identical or partially identical (i.e. at least partially identical) to those of a xylanase derived from the strain Bacillus sp., DSM 7197.

The immunochemical properties can be determined immunologically by cross-reaction identity tests. The identity tests can be performed by the well-known Ouchterlony double immunodiffusion procedure or by tandem crossed immunoelectrophoresis according to Axelsen N.H.; Handbook of Immunoprecipitation-in-Gel Techniques; Blackwell Scientific Publications (1983), chapters 5 and 14. The terms "antigenic identity" and "partial antigenic identity" are described in the same book, chapters 5, 19 and 20.

Monospecific antiserum was generated according to the above-mentioned method by immunizing rabbits with the purified xylanase of the invention. The immunogen was mixed with Freund's adjuvant and injected subcutaneously into rabbits every second week. Antiserum was obtained after a total immunization period of 8 weeks, and immunoglobulin was prepared therefrom as described by Axelsen N.H., supra.

Methods of Producing the Enzymes

The enzyme preparations are obtainable by cultivation of alkalophilic Bacillus sp. in a suitable nutrient medium, containing carbon and nitrogen sources and inorganic salts, followed by recovery of the desired enzyme.

In a preferred embodiment, the enzyme preparations are obtained by cultivation of the alkalophilic species described as Group 3 by Gordon & Hyde [Gordon R.E and Hyde J.L. (1982); Journal of General Microbiology, **128** 1109-1116, Table 4].

In another preferred embodiment, the enzyme preparations are obtained by cultivation of a strain of the alkalophilic species represented by the strain Bacillus sp., DSM 7197.

In a further preferred embodiment, the enzyme preparations are obtained by cultivation of the strain Bacillus sp., DSM 7197, or a mutant or a variant thereof.

The enzyme can also be obtained by recombinant DNA-technology.

The strain Bacillus sp., DSM 7197, was deposited on 4 August 1992 according to the Budapest Treaty on the International Recognition of the Deposits of Microorganisms for the Purpose of Patent Procedures, at Deutsche Sammlung von Mikroorganismen und Zellkulturen, Mascheroder Weg 1b, 3300 Braunschweig, Germany.

Assay for Xylanolytic Activity

The xylanolytic activity is measured in endo-xylanase units (EXU), determined at pH 9.0 with remazol-xylan as substrate.

A xylanase sample is incubated with remazol-xylan substrate. The background of non-degraded dyed substrate is precipitated by ethanol. The remaining blue colour in the supernatant is proportional to the xylanase activity, and the xylanase units are then determined relatively to an enzyme standard at standard reaction conditions, i.e. at 50.0 +/- 0.1°C, pH 9.0, and 30 minutes' reaction time.

A folder AF 293.9/1 describing the analytical method is available upon request to Novo Nordisk A/S, Denmark, which folder is hereby included by reference.

Unless stated otherwise, experiments concerning the effects of temperature and pH on enzyme activity and stability were performed using AZCL-xylan tablets (Xylazyme Tablets™, provided by Megazyme, Australia). The assay was performed as follows:

An appropriate amount of enzyme is dissolved in 1 ml of temperature-equilibrated Britton & Robinson buffer (50 mM). The reaction is started by adding one Xylazyme Tablet, mixed briefly on a Whirley mixer, and incubated at the desired temperature for 15 or 30 minutes. The enzymatic reaction is terminated by addition of 9 ml cold (2-3°C) 1% Tris buffer,

vortexed vigorously, and filtered through a Whatman No. 1 filter circle.

The absorbance of the filtrate is measured at 590 nM. Blank incubations were run in all cases in order to correct for chemical hydrolysis of AZCL-xylan (cf. also Megazyme Product Information Leaflet).

Processes for the Treatment of Lignocellulosic Pulp

In a further aspect, the invention relates to a method for enzymatic treatment of lignocellulosic pulp, comprising employment of an enzyme of this invention.

Enzymatic treatment of lignocellulosic pulp improves the bleachability of the pulp and/or reduces the amount of chemicals necessary for obtaining a satisfactory bleaching.

Due to its temperature stability, the enzyme of the invention may also be applied in a complexing stage of the pulp process, prior to hydrogen peroxide or ozone bleaching.

For use of a xylanase of the invention for delignification of lignocellulosic pulp, the xylanase should preferably be provided in the form of a granulate, preferably a non-dusting granulate, a liquid, in particular a stabilized liquid, a slurry, or a protected enzyme.

In a further preferred embodiment, the agent contains the xylanase in amounts of at least 20%, preferably at least 30%, of the total enzyme protein.

The xylanolytic activity can be measured in xylanase units. In this specification two kinds of units are used: FXU and EXU. By an analytical method a xylanase sample is incubated with remazol-xylan substrate. The background of non-degraded dyed substrate is precipitated by ethanol. The remaining blue colour in the supernatant is proportional to the xylanase activity, and the xylanase units are then determined relatively to an enzyme standard at standard reaction conditions.

The analytical method and the standard reaction conditions are described in two folders: AF 293.6/1 (FXU) and AF 293.9/1 (EXU). FXU is determined at pH 6.0, and EXU is determined at pH 9.0. However, FXU and EXU express enzymatic activity in the same order of magnitude. The folders AF 293.6/1

and 293.9/1 are available upon request to Novo Nordisk A/S, Denmark, which folders are hereby included by reference.

Preferably, the process of the invention is performed at temperatures between 40 and 100°C, more preferred between 50 and 90°C, most preferred between 60 and 80°C.

In another preferred embodiment of the process according to the invention, the enzymatic treatment is performed at a pH above 5.0, more preferred above 6.0, most preferred above 7.0.

In yet another preferred embodiment of the process according to the invention, the enzymatic treatment is performed within a period of 5 minutes to 24 hours, more preferred within 15 minutes to 6 hours, most preferred within 20 minutes to 3 hours.

A suitable xylanase dosage will usually correspond to a xylanase activity of 10 to 5000 FXU/kg or EXU/kg dry pulp, more preferred 100 to 5000 FXU/kg or EXU/kg dry pulp.

In a further preferred embodiment of the process according to the invention, the enzymatic treatment takes place at a consistency of 3-35%, more preferred 5-25%, most preferred 8-15%. The consistency is the dry matter content of the pulp. A pulp with a consistency above 35% is difficult to mix effectively with the enzyme preparation, and a pulp with a consistency below 3% carries too much water, which is a disadvantage from an economic point of view.

In several other preferred embodiments, the xylanases of this invention can be implemented in processes for treatment of lignocellulosic pulp essentially as described in e.g. International Patent Application PCT/DK91/00239, or International Patent Publication WO 91/02839.

Other Applications

The new xylanase enzymes according to the invention may also be well suited for use as baking agents and as additives to animal fodder as described in EP 0 507 723. They may especially be useful for addition to animal feeds for in vivo breakdown of the pentosan fraction as the pH in the small intestine of e.g. poultry, piglets and pigs typically will be

in the area of 5.5 to 7 in which area the new xylanase enzymes have significant activity.

The following examples further illustrate the present invention, and they are not intended to be in any way limiting to the scope of the invention as claimed.

EXAMPLE 1

Cultivation Example

The strain Bacillus sp., DSM 7197, was cultivated at 40°C on a rotary shaking table (300 r.p.m.) in 500 ml baffled Erlenmeyer flasks containing 100 ml of medium of the following composition (per litre):

Xylan (Beechwood)	2.5 g
Yeast extract	5 g
Polypeptone	5 g
NaCl	10 g
K_2HPO_4	1.0 g
$MgSO_4$; $7H_2O$	0.4 g
$CaCl_2$; $2H_2O$	0.1 g
Trace element solution*	2 ml

* Trace mineral solution for Desulfotomaculum acetoxidans medium; Medium 124; DSM Catalogue of Strains 1983.

The medium is sterilized by heating at 120°C for 45 minutes.

After sterilization the pH of the medium is adjusted to 10.0 by addition of approx. 10 ml 1 M sodium sesquicarbonate to each flask.

After 2 days of incubation at 40°C several xylanolytic compounds were produced and excreted into the culture broth.

By isoelectric focusing combined with standard zymogram techniques on gels overlayered with xylan, at least 5

xylanolytic enzymes were detected, having pI in the range of from appr. 3 to appr. 9.5.

EXAMPLE 2

Purification Example

A fraction of xylanases having acidic pI was partially purified by conventional purification techniques involving sample concentration by ultrafiltration and ammonium sulfate precipitation, and conventional chromatographic separation by ionexchange chromatography on S-Sepharose High Load and Q-Sepharose High Load, size exclusion chromatography on Superdex 200 or G-2000 SW, as well as affinity chromatography for specific removal of proteinases.

EXAMPLE 3

Characterization Example

The partially purified enzyme fraction, obtained according to Ex. 2, was subjected to kinetic studies, and the activity was found to be linear (zero-order kinetics) for at least 6 hours, when incubated at the conditions stated in Table 1, below.

Table 1

Relative Vmax* (%)

	40°C	50°C
pH 7.0	-	100
pH 9.0	25	48
pH 10	28	32

* Rate at pH 7.0, 50°C defined as 100%

It appears from the table that the xylanolytic enzymes of the invention exhibit an extraordinary stability and activity even at strong alkaline conditions.

EXAMPLE 4

Characterization Example

The partially purified enzyme fraction, obtained according to Ex. 2, was subjected to experiments concerning the effects of temperature and pH on enzyme activity and stability using AZCL-xylan tablets (Xylazyme Tablets™, provided by Megazyme, Australia). The assay was performed as follows:

An appropriate amount of enzyme is dissolved in 1 ml of temperature-equilibrated Britton & Robinson buffer (50 mM). The reaction is started by adding one Xylazyme Tablet, mixed briefly on a Whirley mixer, and incubated at the desired temperature for 15 or 30 minutes. The enzymatic reaction is terminated by addition of 9 ml cold (2-3°C) 1% Tris buffer, vortexed vigorously, and filtered through a Whatman No. 1 filter circle. The absorbance of the filtrate is measured at 590 nM. Blank incubations were run in all cases in order to correct for chemical hydrolysis of AZCL-xylan (cf. also Megazyme Product Information Leaflet). The results are presented in Figs. 1-4.

The above described fraction was diluted to a concentration of 0.05 EXU/ml in 50 mM Britton & Robinson buffers of pH 7, pH 9, and pH 10, respectively, and incubated at 40°C. At appropriate intervals, 50 µl samples were removed from each incubation mixture and transferred to 950 µl 50 mM Britton & Robinson buffer pH 10. The residual xylanolytic activity was determined at 50°C using Xylazyme Tablets™. The incubation time was 30 minutes in all cases. The results are presented in Figs. 5-7.

EXAMPLE 5

N-terminal amino acid sequence analysis

N-terminal amino acid sequences of the xylanases were determined using standard methods for obtaining and sequencing peptides (Findlay & Geisow (Eds.), Protein Sequencing - a Practical approach, 1989, IRL Press).

The N-terminal amino acid sequence of a xylanase obtained according to ex. 2 and characterized by having a MW of approx. 43 kDa using SDS-PAGE and a pI value of approx. 4.5 in a 3.5 to 9.5 isoelectric focusing gel was found to be (SEQ ID No.1 of the attached sequence listing):

Asn-Asp-Gln-Pro-Phe-Ala-Trp-Gln-Val-Ala-Ser-Leu-

This amino acid sequence is identical to the amino acid sequence of residues 18 to 29 in a 45 kDa xylanase from the alkalophilic Bacillus sp. C-125 (Hamamoto et al., Agric. Biol. Chem. 51, 1987, pp. 953-955).

The N-terminal amino acid sequence of another xylanase purified from the fermentation broth of ex. 1 by conventional chromatographic methods and characterized by having a MW of approx. 22 kDa using SDS-PAGE and a pI value of approx. 9 in a 3.5 to 9.5 isoelectric focusing gel was found to be (SEQ ID No.2 of the attached sequence listing):

Asn-Thr-Tyr-Trp-Gln-Tyr-Xaa-Thr-Asp-Gly-Gly-Gly-Thr-Val-Asn-Ala-Xaa-Asn-Gly-

Xaa designates unidentified residues. This amino acid sequence is homologous to some of the other low molecular weight xylanases characterized so far (e.g. the xylanase from Bacillus subtilis, for reference see Paice et al., Arch. Microbiol. 144, 1986, pp. 201-206).

EXAMPLE 6

Mass Spectrometry

Matrix assisted lase desorption ionisation time-of-flight mass spectrometry was carried out using a ToftSpec™ mass spectrometer from VG Analytical according to the manufacturers instructions.

Matrix assisted lase desorption ionisation time-of-flight mass spectrometry gave a mass value of 20532 Da ±0.1% for the xylanase described in ex.5 having a MW of approx. 22 kDa and a pI of approx. 9.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: NOVO NORDISK A/S
(B) STREET: Novo Alle
(C) CITY: DK-2880 Bagsvaerd
(E) COUNTRY: Denmark
(F) POSTAL CODE (ZIP): DK-2880
(G) TELEPHONE: +45 44 44 88 88
(H) TELEFAX: +45 44 49 32 56
(I) TELEX: 37304

(ii) TITLE OF INVENTION: NOVEL ENZYMES

(iii) NUMBER OF SEQUENCES: 2

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(v) CURRENT APPLICATION DATA:
APPLICATION NUMBER:

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Asn Asp Gln Pro Phe Ala Trp Gln Val Ala Ser Leu
1 5 10

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Asn Thr Tyr Trp Gln Tyr Xaa Thr Asp Gly Gly Gly Thr Val Asn Ala
1 5 10 15

Xaa Asn Gly

International Application No: PCT/ /

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 2, line 2 of the description [1]

A. IDENTIFICATION OF DEPOSIT [4]

Further deposits are identified on an additional sheet ☐ [5]

Name of depositary institution [6]

DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELL-KULTUREN GmbH

Address of depositary institution (including postal code and country) [6]

Mascheroder Weg 1b, D-3300 Braunschweig, Federal Republic of Germany

Date of deposit [6]	Accession Number [6]
4 August 1992	DSM 7197

B. ADDITIONAL INDICATIONS [7] (leave blank if not applicable). This information is continued on a separate attached sheet ☐

In respect of those designations in which a European patent is sought, a sample of the deposited microorganism will be made available only by the issue of such a sample to an expert nominated by the person requesting the sample (Rule 28(4) EPC) until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or is deemed to be withdrawn.

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE [8] (if the indications are not for all designated States)

D. SEPARATE FURNISHING OF INDICATIONS [9] (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later [9] (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. ☐ This sheet was received with the international application when filed (to be checked by the receiving Office)

Susanne Fjeldsted
(Authorized Officer)

☐ The date of receipt (from the applicant) by the International Bureau [10]

was

(Authorized Officer)

CLAIMS

1. An enzyme preparation having xylanolytic activity and **characterized** by having the following properties:

(a) more than 50% relative activity in the range pH 6-9 at 50°C;

(b) temperature optimum in the range of from 55 to 75°C (at pH 6-10); and

(c) immunochemical properties identical or partially identical to those of a xylanase derived from the strain Bacillus sp., DSM 7197.

2. An enzyme preparation according to claim 1, the enzyme preparation being obtainable from a strain of Bacillus sp., or from another host organism carrying the gene encoding a xylanase having immunochemical properties identical or partially identical to those of the xylanase derived from the strain Bacillus sp., DSM 7197.

3. An enzyme preparation according to either of claims 1-2, the enzyme preparation being obtainable from a strain belonging to the species represented by the strain Bacillus sp. DSM 7197.

4. An enzyme preparation according to any of claims 1-3, the enzyme preparation being obtainable from the strain Bacillus sp., DSM 7197, or a mutant or a variant thereof.

5. A process for the preparation of an enzyme preparation according to any of claims 1-4, which process comprises cultivation of a strain of Bacillus sp., preferably the strain Bacillus sp., DSM 7197, or a mutant or a variant thereof, in a suitable nutrient medium, containing carbon and nitrogen sources and inorganic salts, followed by recovery of the desired enzyme.

6. The use of the enzyme preparation according to any of claims 1-4 in a process for treatment of lignocellulosic pulp.

7. A process according to claim 6 for treatment of lignocellulosic chemical pulp, wherein the lignocellulosic pulp is treated with the enzyme preparation at a pH above 6.5, preferably above 7.5, whereafter the thus treated cellulosic pulp is treated with chlorine at an active chlorine multiple of 0.20 or less in the first chlorination stage.

8. The use of the enzyme preparation according to any of claims 1-4 in a process for treatment of animal feed.

9. The use of the enzyme preparation according to any of claims 1-4 as a baking agent in the production of bread.

10. An agent containing an enzyme preparation according to any of claims 1-4, provided in the form of a granulate, preferably a non-dusting granulate, a liquid, in particular a stabilized liquid, a slurry, or a protected enzyme.

11. An agent according to claim 10, in which the xylanase preparation constitutes at least 20%, preferably at least 30%, of the total enzyme protein.

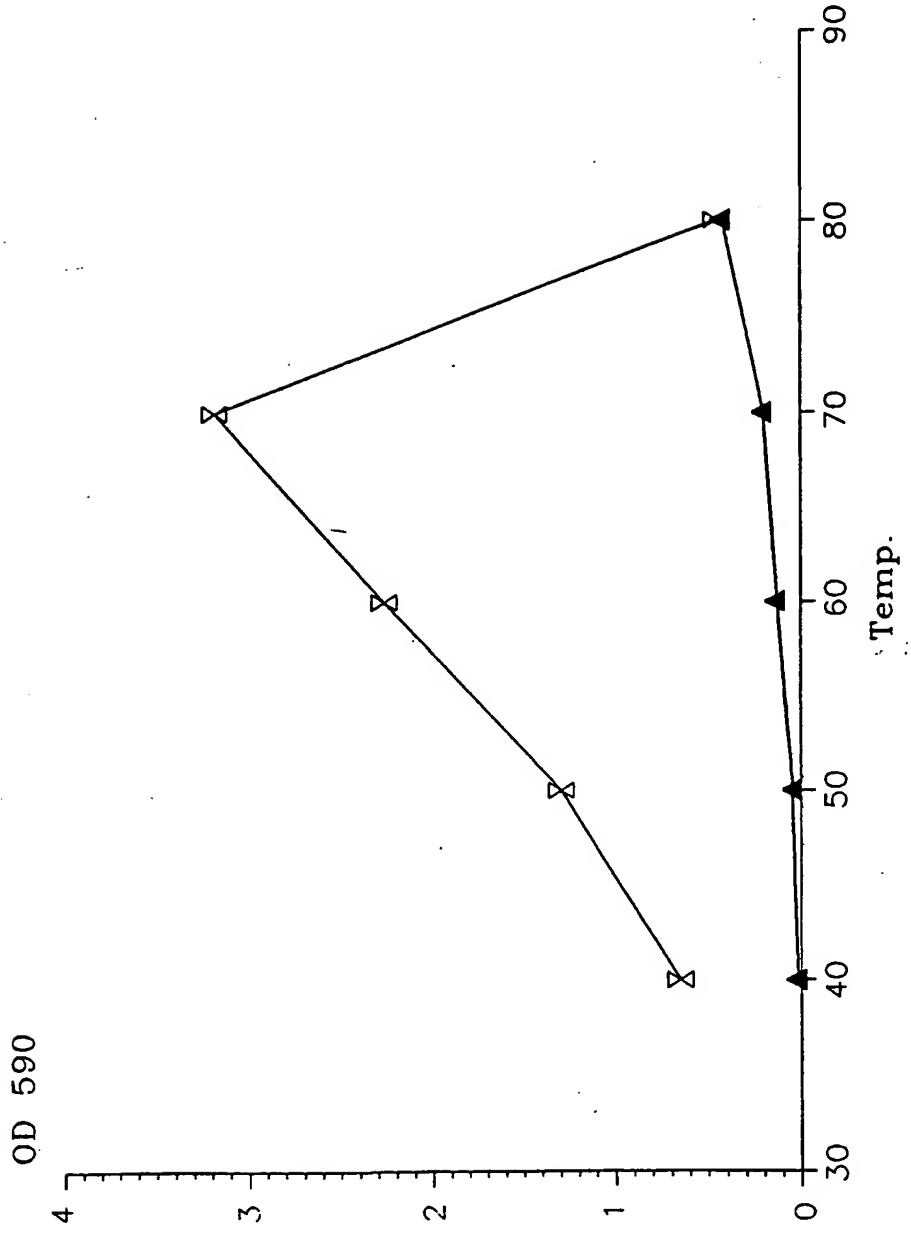

Fig. 1

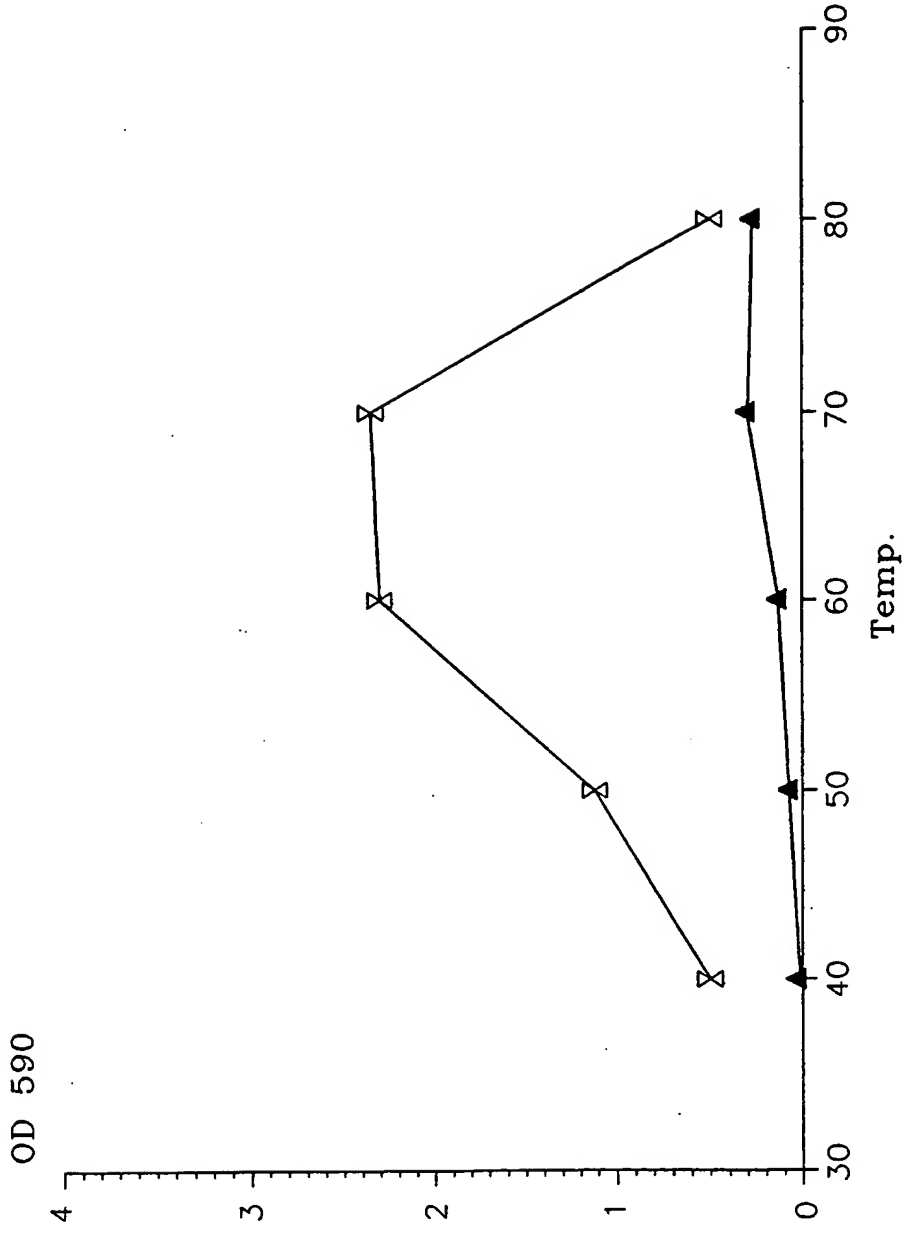

Fig. 2

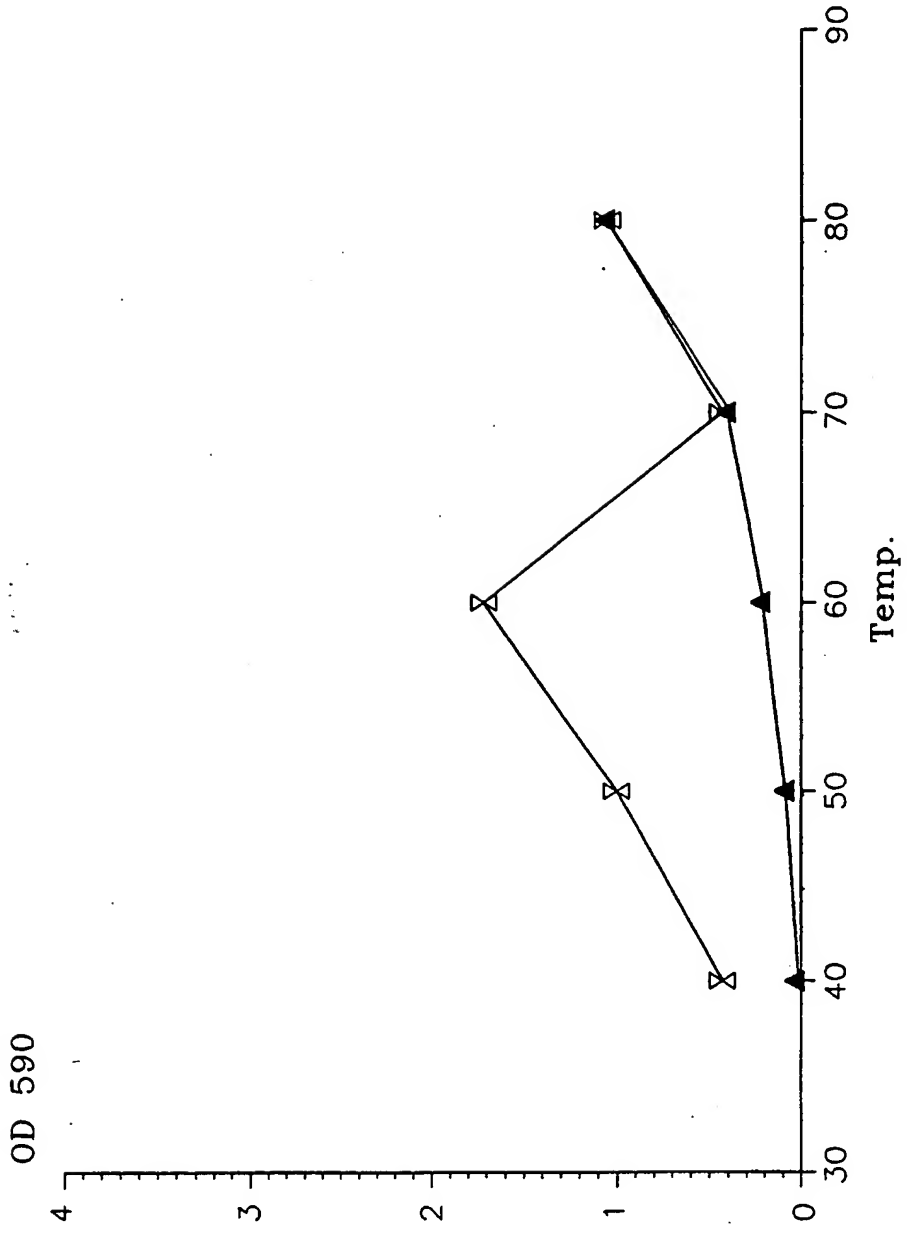

Fig. 3

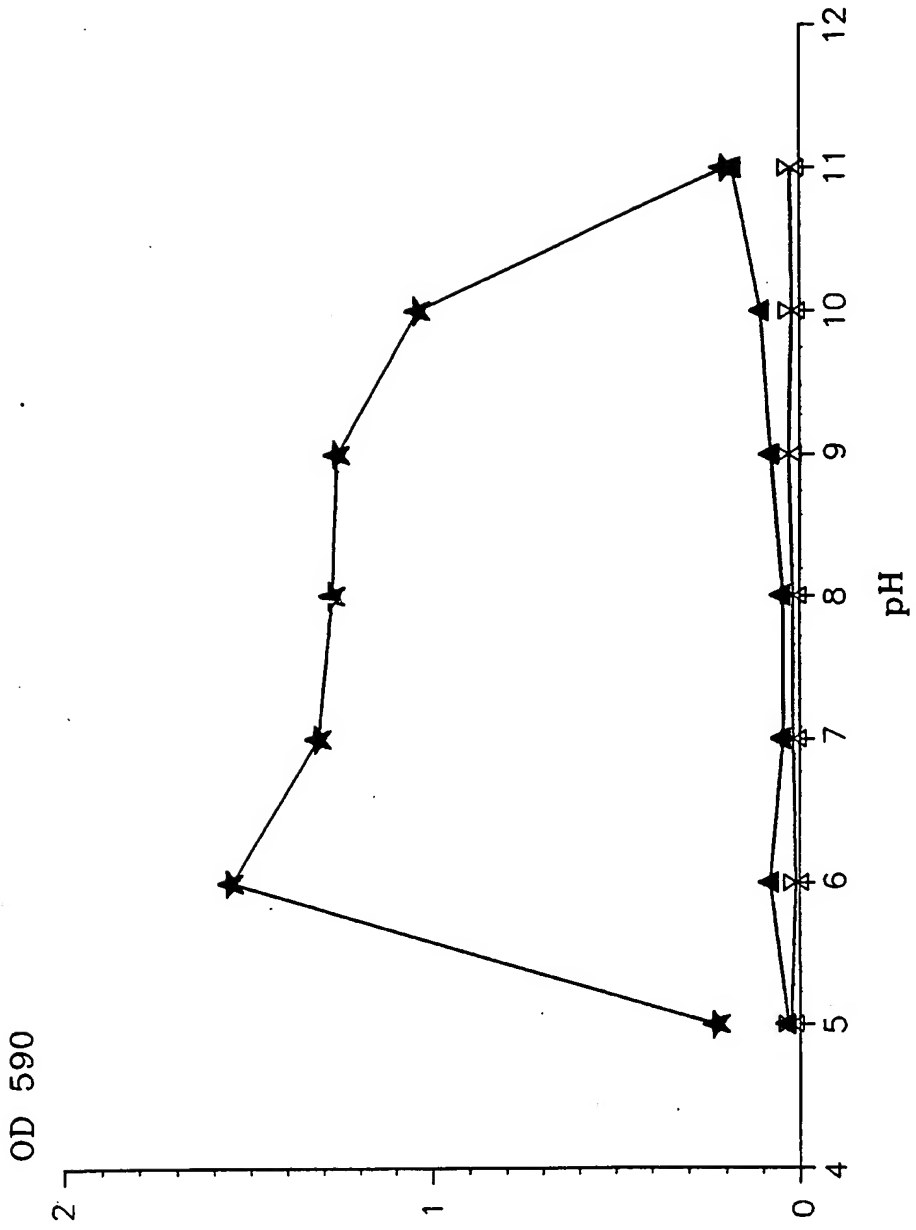

Fig. 4

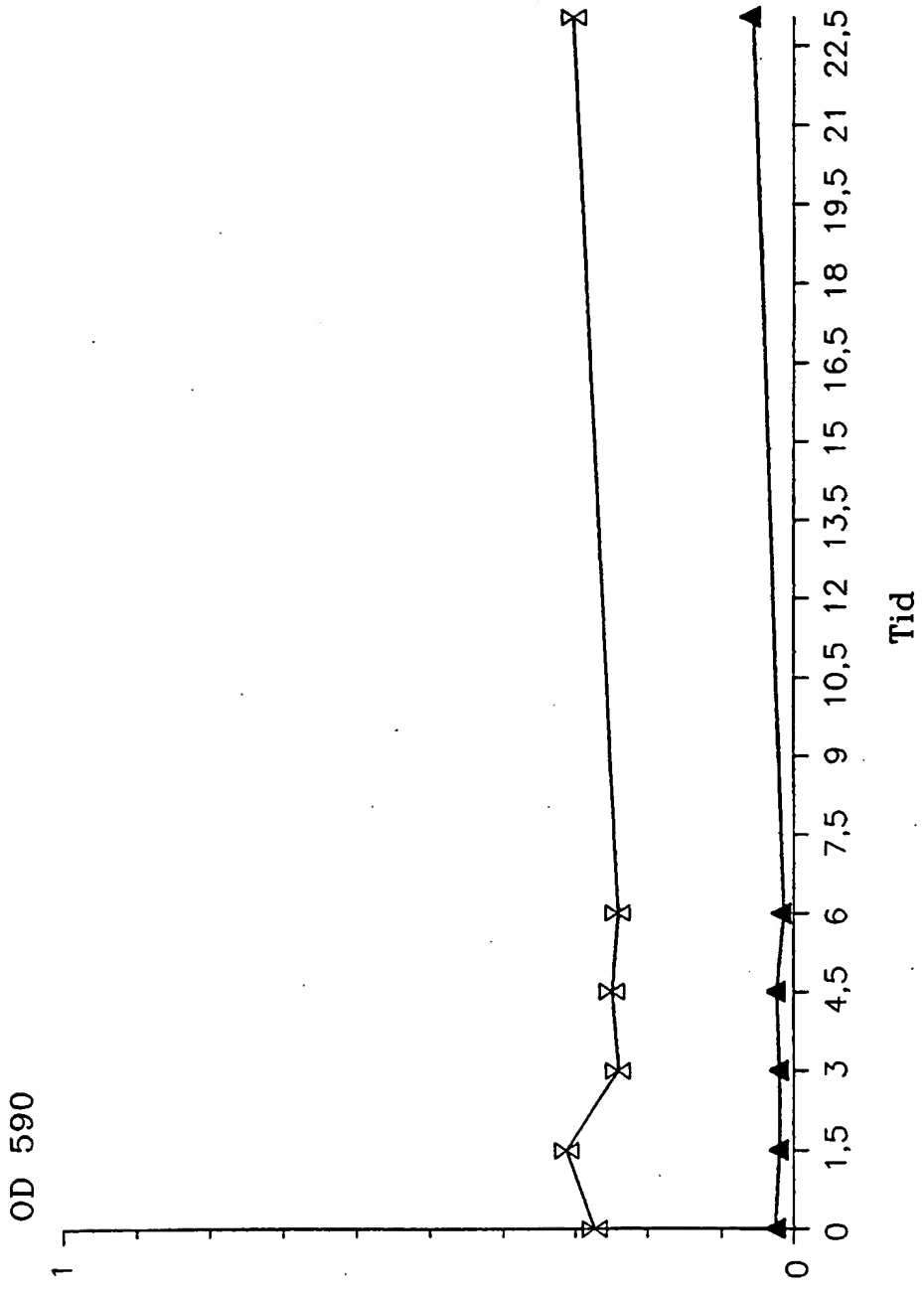

Fig. 5

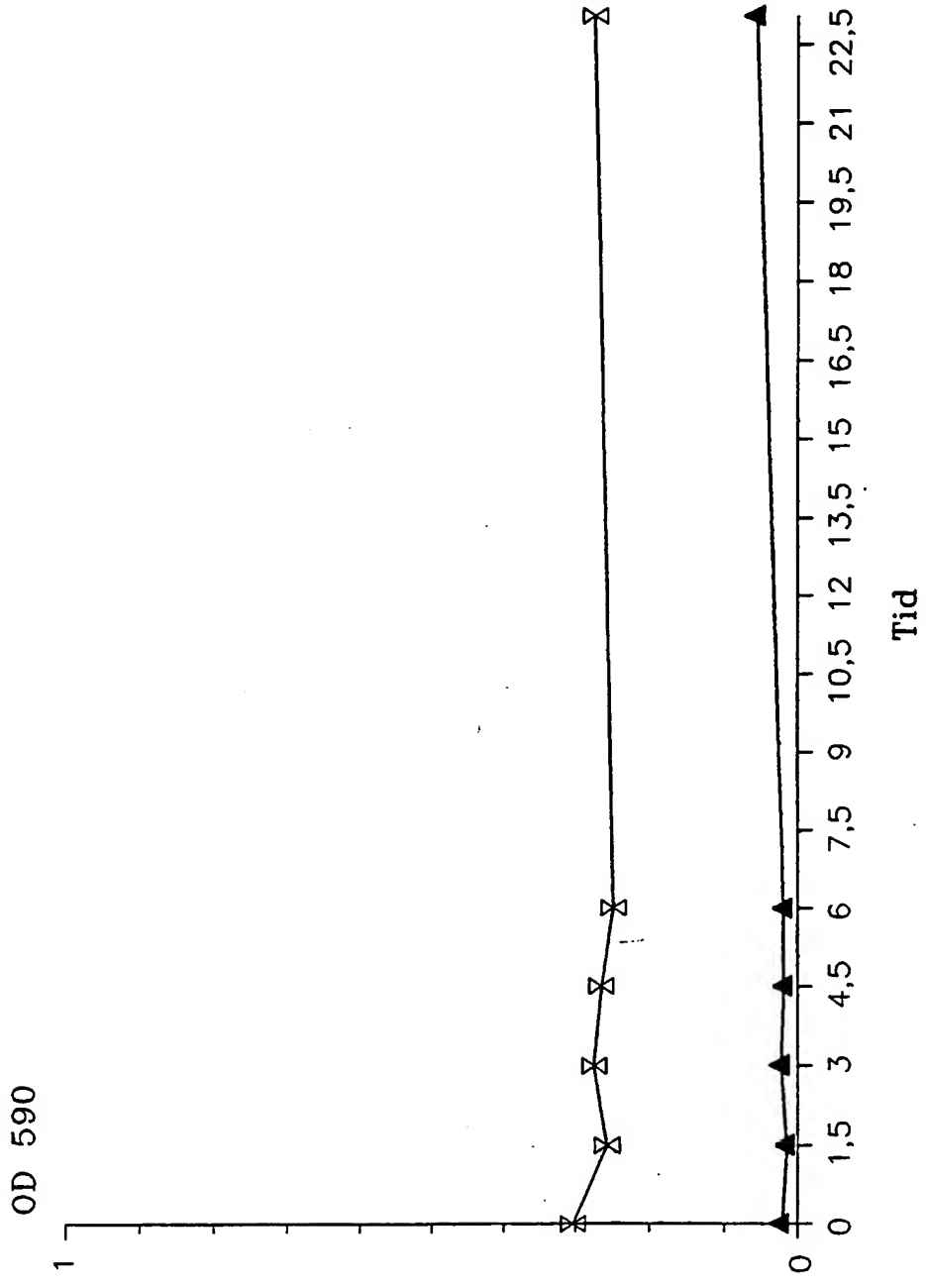

Fig. 6

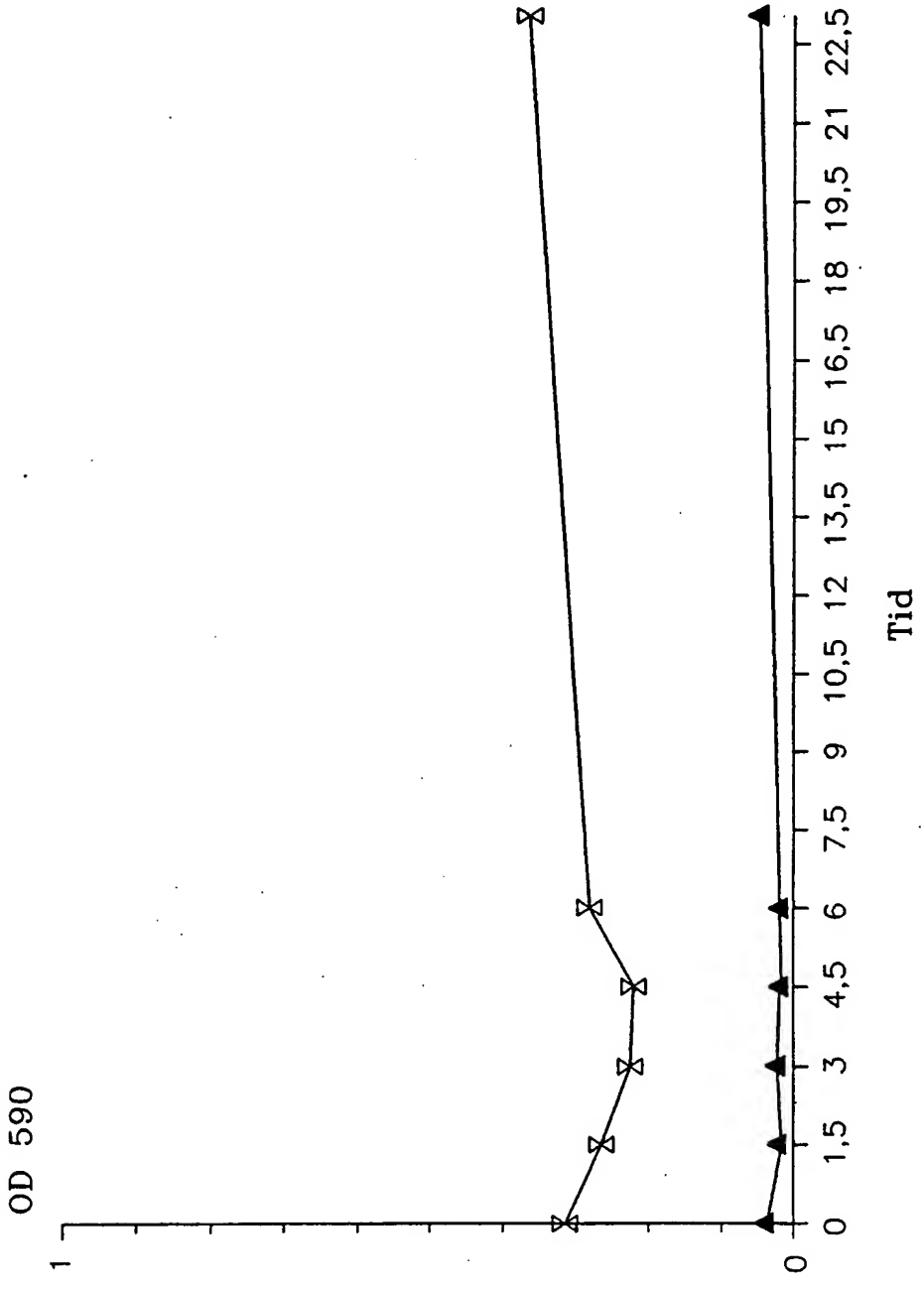

Fig. 7

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 93/00277

A. CLASSIFICATION OF SUBJECT MATTER

IPC5: C12N 9/24, C12S 3/08
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC5: C12N, C12S

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO, A1, 9118976 (NOVO NORDISK A/S), 12 December 1991 (12.12.91), claim 15	1-7,10-11
	--	
X	WO, A1, 9110724 (KORSNÄS AB), 25 July 1991 (25.07.91)	1-7,10-11
	--	
P,X	BIOTECHNOLOGY LETTERS, Volume 14, No 11, November 1992, N. Gupta et al, "A thermostable extracellular xylanase from alkalophilic bacillus sp. NG-27", figures 1-2	1-6,10-11
	--	

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 November 1993	30 -11- 1993
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Mikael G:son Bergstrand Telephone No. +46 8 782 25 00

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INTE[redacted]IONAL SEARCH REPORT

[redacted]tional application No.
PCT/DK 93/00277

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	AGRIC. BIOL. CHEM., Volume 51, No 3, 1987, Tetsuo Hamamoto et al, "Nucleotide Sequence of the Xylanase A Gene of Alkalophilic Bacillus sp. Strain C-125", Fig. 2 around nucleotide 150 --	1-6,10-11
X	AGRIC. BIOL. CHEM., Volume 49, No 11, 1985, Hiroshi Honda et al, "Purification and Partial Characterization of Alkaline Xylanase from Escherichia coli Carrying pCX311", figures 4 and 5 -- --------	1-6,10-11

INTERNATIONAL SEARCH REPORT
Information on patent family members

01/10/93

International application No.
PCT/DK 93/00277

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A1- 9118976	12/12/91	NONE	
WO-A1- 9110724	25/07/91	AU-B- 631485	26/11/92
		AU-A- 7166391	05/08/91
		SE-B,C- 465320	26/08/91
		SE-A- 9000070	11/07/91